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Final report of the land-based testing of the Ecochlor®-System, for Type Approval according to Regulation-D2 and the relevant IMO Guideline

(April – July 2008)

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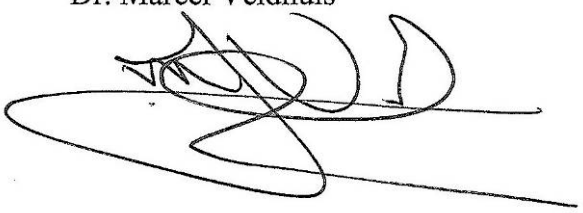

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Content page

1 Executive summary

The ECOCHLOR®-System, a ballast water treatment system, was tested according to the Regulation-D2 (D2-Standard), the IMO Guidelines for Type Approval testing (G8) and for approval of BWT systems that make use of active substances (G9) in the spring and early summer of 2008 in the harbour of the Netherlands Institute for Sea Research.

In general most of the requirements for testing were met and in several test runs environmental conditions were harsher than strictly required. The original ECOCHLOR®-System was designed without a mechanical filtration step but the system performed much better with the addition of a coarse filter (BSFc, 40 micron filter). In total 11 test runs were successful and the BWT system performed on average much better than stated in Regulation-D2 by achieving values for organisms well below the requirements of the D2 Standard. For many size classes of organisms the residual number of organisms was at least one order of magnitude lower than indicated numbers in the Standard-D2. This system should therefore be regarded as an effective way of cleaning ballast water in ships, thereby minimizing the risk of new invasions originating from the ships ballast water.

The sediment load was reduced by the self-cleaning filter and organisms were killed by the active substance chlorine-dioxide. Except for bacteria nearly all organisms disintegrated completely and the remaining debris was found as an amorphous structure or as dissolved organic carbon.

No regrowth was observed for a period exceeding at least 15 days. Environmental acceptability tests however showed that the growth of organisms, mainly plankton, was not limited by the discharge water indicating that the discharged water was still vital.

Upon discharge no residual effects of the chemical treatment to the receiving environment were observed. The active substance decomposed within the treated ballast tank.

2 Zusammenfassung

Das ECOCHLOR®-System zur Behandlung von Ballastwasser wurde im Frühjahr und Sommer 2008 gemäß Regularien-D2 (D2-Standard), sowie der IMO Richtlinien zu Tests für eine Typzulassung (G8) und denen für eine Zulassung von Systemen, welche aktive Substanzen verwenden (G9) im Hafen des Königlich Niederländischen Meeresforschungsinstitut (NIOZ) getestet.

Generell wurden die allermeisten Anforderungen bezüglich der abiotischen Parameter des Testwassers und zur Organismendichte erfüllt. In den meisten Tests waren die Bedingungen sogar schwieriger, als in den Richtlinien verlangt. Das ECOCHLOR®-System war ursprünglich ohne mechanische Filtration entworfen, aber das System arbeitete deutlich besser nach dem Zufügen eines Feststofffilters (BSFc, 40 Mikrometerfilter). Es wurden 11 voneinander unabhängige Tests nacheinander, erfolgreich durchgeführt. Das System erfüllte alle Anforderungen der Regularien-D2 und der Richtlinie G8. Die Organismenanzahlen nach der Behandlung lagen deutlich unter den Anforderungen des D2-Standards, für einige Gruppen im Durchschnitt sogar um eine Größenordnung. Das System sollte demzufolge als effektive und sichere Möglichkeit zur Behandlung von Ballastwasser betrachtet werden, die dazu beitragen kann, weitere biologische Invasionen zu verhindern.

Die Sedimentfracht wurde durch den Vorbehandlungsschritt reduziert (selbstreinigender Filter) und die Organismen durch die aktive Substanz Chlordioxid inaktiviert. Mit der Ausnahme von Bakterien wurden fast alle Organismen vollständig zersetzt. Die Überreste der Organismen fanden sich lediglich in Form amorpher Strukturen und als gelöster organischer Kohlenstoff.

In Inkubationsexperimenten über Zeiträume von mehr als 15 Tagen unter vorteilhaften Umweltbedingungen konnte kein Planktonwachstum im behandelten Wasser festgestellt werden. Gleichzeitig durchgeführte Umweltverträglichkeitstests (Zugabe von Zeigeorganismen und Planktonkulturen, Verdünnungsreihen) mit dem behandelten Wasser ergaben jedoch keine negativen Auswirkungen auf das Planktonwachstum und zeigten somit, dass das behandelte Wasser selbst das Wachstum gesunder Organismen nicht beeinflusst.

Bei der Abgabe des behandelten Wassers wurden keine negativen Effekte auf den empfangenden Wasserkörper festgestellt. Die aktive Substanz wurde in den Tanks gemäß der Erwartungen aus den Vorversuchen abgebaut.

3 Summary table with results for the Type Approval Certificate of the ECOCHLOR® BWT-System

Land-based tests NIOZ	**Reference & Treated**			**Reference**			**Treated**		
salinity 31.9 PSU	**Intake**			**Discharge**			**Discharge**		
natural plankton	Average	min.	max.	Average	min.	max.	Average	min.	max.
total bacteria [counts/mL]	3.79+E6	1.25+E6	5.42+E6	2.15+E6	0.33+E6	5.37+E6	4.90+E6	0.48+E6	8.24+E6
E. coli [cfu/mL]	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1
Enterococci [cfu/mL]	5.0	< 1	17	< 1	< 1	1	< 1	< 1	< 1
plankton <10 µm [counts/mL]	2264	1004	4556	290	173	632	1.0	n.d	3
plankton 10-50 µm [counts/mL]	1628	730	3207	140	121	151	0.7	n.d.	3.7
plankton >50 µm [counts/m^3]	1.78+E5	0.52+E5	4.11+E5	1.54+E4	0.25+E4	2.76+E4	0.9	n.d.	3.7

Land-based tests NIOZ	**Reference & Treated**			**Reference**			**Treated**		
salinity 23,1 PSU	**Intake**			**Discharge**			**Discharge**		
natural plankton	Average	min.	max.	Average	min.	max.	Average	min.	max.
total bacteria [counts/mL]	6.47+E6	3.66+E6	9.02+E6	1.89+E6	0.86+E6	2.90+E6	4.74+E6	2.54+E6	7.86+E6
E. coli [cfu/mL]	0.5	< 0,1	1.4	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1	< 0,1
Enterococci [cfu/mL]	9.3	< 1	27	< 1	< 1	1	< 1	< 1	< 1
plankton <10 µm [counts/mL]	8945	3278	15096	712	393	1195	1.6	n.d.	6.7
plankton 10-50 µm [counts/mL]	1326	719	1746	157	130	175	0.1	n.d.	0.7
plankton >50 µm [counts/m^3]	1.76+E5	0.36+E5	3.61+E5	6.55+E3	2.7+E3	8.57E3	n.d.	n.d.	n.d.

Summary table of collected data covering the major groups of organisms at two series of 6 and 5 test-runs for low and high salinity range, respectively.
n.d.: non detectable in sample.

4 Acknowledgements

The authors thank the technical staff of the NIOZ and in particular Anna Noordeloos, Eveline Garritsen, Swier Oosterhuis, Siem Groot, Robert Lakeman, Santiago Gonzalez and Pieter van Kralingen. Their cooperation was essential for the successful completion of this certification project.
Special thanks also to Yolanda van Iperen for providing the species list of plankton in the Wadden Sea.
We also thank Mr. Rolf von Ostrowski, Dr. Kai Trümpler and Mrs. Karin Sigler of the Bundesambt für Seeshifffahrt und Hydrographie, acting on behalf of the German Administration, for their excellent collaboration during this whole certification process.

5 Introduction

Ships transport 5-10 billion tons of ballast water annually all over the globe (Endresen *et al.*, 2004). The ballast water is loaded with particulate sediment and an enormous variety of (living) organisms, which ranges from juvenile stages, larvae and eggs of fish and larger zooplankton (Williams *et al.*, 1988); (Carlton and Geller, 1993) to macroalgae, phytoplankton (Hamer *et al.*, 2000; Hallegraeff *et al.*, 1997), bacteria and viruses (Gollash *et al.*, 1998). In general these organisms belong to the natural ecosystem in and around the port of origin but they might not be occurring naturally in the coastal waters and port of destination at the end of a ship's journey. In hundreds of cases around the world, this has resulted in severe damage to the receiving ecosystem and to human health, because these non-native organisms developed into a plague. This often has a high impact on the ecosystem and can cause economical damage (Hoagland *et al.*, 2002), as it results in a decrease of stocks of commercially valuable fish and shellfish species and occasionally outbreaks of diseases such as cholera (Ruiz *et al.*, 2000; Drake *et al.*, 2001). If action is not taken the problem of invasive species will increase in an exponential manner for several reasons. Ships are getting larger, faster and the amount of traffic across the oceans is expected to increase rapidly during the coming decades, and therefore also the change of non-indigenous organisms to have large enough numbers for settling and expanding. Our effort to reduce pollution of ports and coastal waters also improves the quality of the aquatic environment in these areas and therefore increases the susceptibility to invasive organisms. Originally not intentionally meant but organisms in ballast water will experience favourable conditions for settling and growing. The problem of invasive species is considered as one of the 4 major threats of the world's oceans next to land-based marine pollution, overexploitation of living marine resources, and physical alteration/destruction of habitats

To minimize these risks for the future, the International Maritime Organization (IMO) of the United Nations has adopted the Ballast Water Convention in 2004 (Anonymous, 2005). The Convention states that finally ALL ships (>50,000 in number) should install proper ballast water treatment (BWT) equipment on board between 2009 and 2016. As a temporary and intermediate solution for the time being ship may reduce the risk of invasive species by performing ballast water exchange during their voyage when passing deep water (>200 m depth and 200 NM from the coast. Ballast water exchange faces many problems as to feasibility, safety and efficacy For a large part of ships' voyages the required depth and/or distance to shore requirements are never met; BW exchange can affect the ships construction stability and in rough seas exchange is not possible because of the risk to ship and crew. Treatment of ballast water is therefore considered to be the best solution of reducing the risk of invasive species.

During the recent years numerous solutions for treatment of ballast water have been mentioned and tested with the ultimate goal to reduce the amount of organisms in ballast water (Rigby and Taylor, 2001). However, next to a high efficacy there is more needed for a BWT system to be good a system. Next to biologically effective the system should be practicable, environmentally acceptable and also cost effective.
Despite the fact that the treatment technology for drinking-, waste- and process water is well-developed none of these techniques is directly applicable to ballast water (Rigby *et al.*, 2001; MEPC 49/2/13, 2003). Besides reducing the load of organisms the sediment load should be reduced as well. There are also considerable differences in ships operation, types of ships, and the amount of space available for a ballast water treatment system on board and the way ships are operated. Ballast water treatment will develop into a new field of technology of its own with a commercial market estimated for the next 10 years in the order of 8 billion Euro (Haskoning, 2001).
As a primarily scientific research institute NIOZ is defining its role in the certification process as to study

1) the numerical abundance and biodiversity of organisms prior, during and after a treatment with the ECOCHLOR Ballast Water Treatment system (efficacy of the BWT system),
2) to determine the viability status of the remaining organisms during discharge,
3) to assess possible environmental risks of discharging chlorine-dioxide-treated ballast water by measuring residual effluent toxicity in order to determine latent effects, other than measured in specific toxicity tests conducted for the G9 (environmental impact).

This research strategy allows for more in depth testing, while it includes ALL organisms and not only the size classes as specified in the Convention D2-standard.

6 Description of the treatment facility

6.1 NIOZ Royal Netherlands Institute for Sea Research

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is part of the Netherlands Organization for Scientific Research (NWO). The institute employs around 200 people and the annual budget is approximately €20 million.
The mission of NIOZ is to gain and communicate scientific knowledge on seas and oceans for the understanding and sustainability of our planet. The institute also facilitates and supports marine research and education in the Netherlands and in Europe.

In order to fulfil its mission, the institute performs tasks in three specific fields.
Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. The institute also carries out research based on societal questions when this merges well with its fundamental work. The senior scientists at NIOZ all participate in international research projects.
Education: The institute educates PhD and other students of universities and schools for professional education. Together with universities NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists of NIOZ is also appointed as professor at Dutch and foreign universities.
Facilitary services: NIOZ invites marine scientists from Dutch and foreign institutes and universities to write scientific proposals involving the institute's research vessels, laboratories, and the large research equipment, which is often designed and built by the institute's own technical department.
The basic oceanographic **disciplines** studied at NIOZ are physics, chemistry, biology and geology. Multidisciplinary research is regarded as one of the main strengths of NIOZ.

More information on www.nioz.nl

Figure 1: aerial view of the NIOZ harbour (lower right), NIOZ laboratories (upper left) and TESO ferry (top).

6.2 Portrait of ECOCHLOR (producer of the ECOCHLOR-system)

More information on: www.ecochlor.com

Ballast water treatment - The ECOCHLOR-System
The ECOCHLOR®-System operates in-line during the uptake of the ballast water. It is based on a two-step treatment process which includes physical separation (self-cleaning filter) as well as secondary treatment with chlorine-dioxide as the active substance.

The Ecochlor® BWTS is viable for all ballast water capacity ranges, and is distinguished by the system's capability to treat at high ballast water flow rates. The Ecochlor® BWTS can be installed as a retrofit on existing vessels or during a new construction, with the latter being the most straightforward and cost effective. Additionally, Ecochlor's treatment system can be easily incorporated into a land-based system to treat ballast water land-side.

Ecochlor, Inc., www.ecochlor.com, founded in 2001, has developed proprietary ballast water treatment systems that have been specifically designed to safely and economically eliminate the worldwide transfer of aquatic invasive species. Ecochlor's technology is patented, proven, well understood and uniquely competitive. The company has an experienced management team and has established key partnerships in naval architecture, marine engineering, systems engineering, fabrication, science, chemicals, distribution, and service. Ecochlor is based in the United States with capabilities to install, service, and support its systems throughout the world.

In research underwritten by Ecochlor at the University of Rhode Island, Graduate School of Oceanography (URI), the use of chlorine dioxide was proven effective in seawater on representative groups of all target organisms. Additional extensive shipboard testing has been conducted by URI under a grant from the United States National Oceanographic and Atmospheric Administration (NOAA).

Ecochlor has installed two commercial systems to date. In July 2004, the company installed its first system on Atlantic Container Lines *Atlantic Compass*, one of the world's largest combined Container/RORO ships. This ship is a Swedish flagged vessel that carries cargo between Europe and North America. In July 2005, the company installed its second system on the bulker M/V *Moku Pahu*, operated by Matson Navigation that carries sugar from Hawaii to the West Coast, USA and grain to Asia.

International approval is mandatory, and Ecochlor has undertaken a two-pronged global approach to certification. In the United States, the two Ecochlor systems installed onboard have been accepted into the U.S. Coast Guard (USCG) Shipboard Technology Evaluation Program (STEP). European countries are ahead of the U.S. in the approvals process for ballast water treatment systems. Ecochlor was granted IMO Basic Approval in July 2008, and an application for IMO Final Approval has been submitted. Ecochlor is pleased to work with the Royal Netherlands Institute for Sea Research (NIOZ) to conduct certification testing and the German Federal Maritime Agency (BSH) who is acting as the administrating agency during the certification and Type Approval process.

6.3 The test facility

The land-based tests were carried out at the Royal Netherlands Institute for Sea Research (NIOZ, www.nioz.nl), Landsdiep 4, 1797 SZ 't Horntje, Texel, the Netherlands, from March through July 2008.

The NIOZ test site is equipped with 3 coated concrete tanks of 300 m³ volume each to simulate the ballast water tanks of the ship (Figure 2). The tanks were cleaned using pressure-washing after each run. Water samples were taken from bypasses of the standard piping used to fill and to empty the tanks or directly from the tank at outflow at ca. 1 m from the bottom.

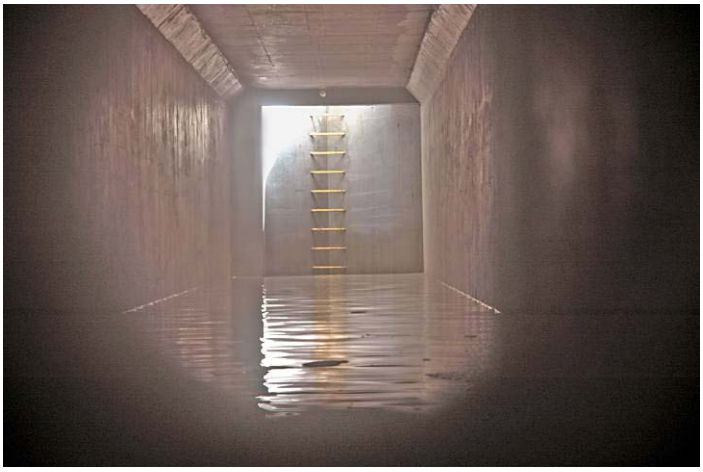


Figure 2: Inside view of one of three subterranean water tanks

The Ecochlor-BWT-System was connected to a typical ballast water pump which was located in the NIOZ harbour. This is a pristine harbour with a direct access to the Wadden Sea and the origin of the test water changes with the tide. Furthermore, provisions were made to allow the addition of salt water and / or freshwater in order to adjust the salinity of the natural water of the NIOZ harbour to the required test conditions of brackish water and marine water with a minimum of 10 PSU difference. A detailed description of the test installation is presented in figure 3.

According to the requirements of the Guidelines G8, sampling points are fitted before the treatment system and directly after the system. Samples varying in volume from 500 mL up to 1 m^3 were taken using clean sampling containers. Sampling containers and all further handling of the samples were separated in a control and a treated set to avoid cross contamination by the active substance. The basic handling, such as concentrating, filtration and chemical analysis was done at the test site. Additional samples (1 to 10 L) were transported to the institute's laboratories for further special analysis. For re-growth experiments 10 L of sample was transported (Nalgene bottle) to a climate room for incubation experiments (ca. 12 – 15 °C; a light; dark regime of 16:8 h and 100 µmol quanta. $m^{-2}.s^{-1}$)

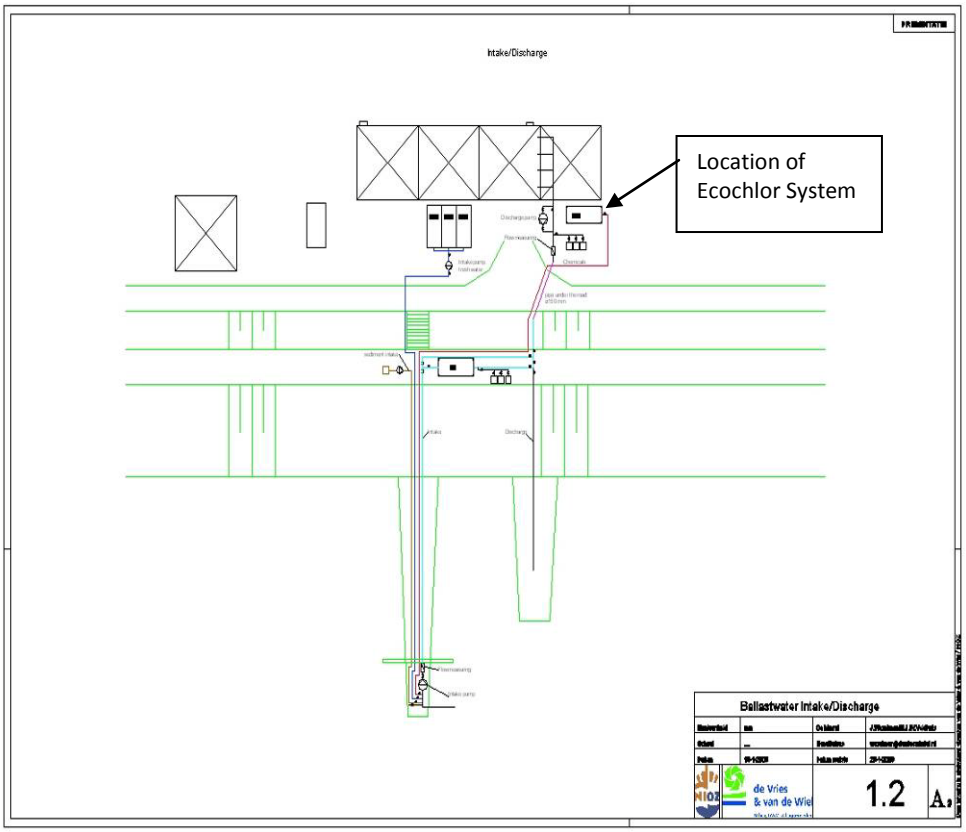


Figure 3: P&I diagram of the Ecochlor installation at NIOZ with points of sampling

6.4 Technical description of the ECOCHLOR-System

The Ecochlor® Ballast Water Treatment System (BWTS) applies established chlorine dioxide technology to oxidize and disinfect aquatic invasive species (AIS). Chlorine dioxide has a long history (50+ years) of being applied safely and economically for the control of microorganisms in industrial and municipal waters. What is unique about chlorine dioxide is its ability to be effective against a wide range of troublesome organisms. A second unique feature of chlorine dioxide as compared to other oxidants (such as chlorine) is the minimization of unwanted by-products. For example, chlorine reacts by both an oxidative and a substitution pathway. Because of this, unwanted chlorinated by-products such as trihalomethanes (THMs) typically accompany chlorine applications. In contrast, chlorine dioxide reacts only by an oxidative pathway thereby eliminating the potential to form unwanted and regulated chlorinated organic by-products even in the most heavily contaminated waters.

The Ecochlor® BWTS generates chlorine dioxide using the Eka Chemicals Purate® technology, a patent-protected chlorine dioxide generation method. Eka's Purate® technology is a chlorate-based chlorine dioxide generation process. This method differs from other conventional chlorine dioxide generation methods that involve use of aqueous or gaseous chlorine, making the Purate® method environmentally superior. Additionally, Eka's Purate® technology produces chlorine dioxide efficiently, thereby controlling the required input of precursor chemicals and eliminating production of unwanted by-products.

The Ecochlor® BWTS generates and injects a dilute solution of chlorine dioxide during the uptake of ballast water. Chlorine dioxide is not stored as a concentrated chemical at any

time. It is generated automatically on demand based on the amount of ballast water being pumped. After injection into the incoming ballast water, chlorine dioxide remains effective in the ballast water tanks for a short period (2 – 20 hours) in order to neutralize ballast tank biofilm that can cause bacterial re-growth. Over time, the chlorine dioxide continues to react and/or decay in the ballast tanks so that at the time of discharge the ballast water contains an undetectable concentration of chlorine dioxide.

Prior to treatment, the water is filtered through a 40 micron BallastSafe™ BSFc Automatic Electric Filter, Model BSFc-H-1.6. The unique sintered stainless steel screen technology enables it to have an unparalleled zooplankton removal rate. BallastSafe's filter features continuous cleaning of large volumes of sediment during ballasting without interruption, and a reversible screw system for smooth, reliable and rapid cleaning of the entire screen surface. The effectiveness of this filter has been successfully demonstrated in previous trials at NIOZ.

During all test cycles, with the exception of high salinity test run 6, chlorine dioxide treatment was applied to incoming ballast water at 5 mg/L. During test cycle 6, chlorine dioxide was applied at a dose of 4 mg/L. This was the first test run after the addition of the filtration phase and Ecochlor wanted to determine the effectiveness of 4 mg/L treatment. Although the IMO D-2 standard was met using 4 mg/L, this treatment was not enough to demonstrate compliance with more stringent standards that are being proposed in the United States. Therefore, the remaining test cycles were performed using a 5 mg/L dose of chlorine dioxide.

The Ecochlor® Ballast Water Treatment System at NIOZ for land based testing is Ecochlor's smallest commercial system and has not been downsized. The Ecochlor® BWTS is a self-monitoring system and has been reviewed for compliance with the class requirements of the Lloyd's Register North America and ABS Americas Division classification societies. All materials and specifications were selected in order to meet the class requirements of these classification societies for ballast water systems and electric installations.

Subsequently, the prototype Ecochlor® BWTS that is installed on two test ships (M/V *Atlantic Compass* and M/V *Moku Pahu*) have received full plan approval. The *Atlantic Compass* system was approved by Lloyd's Register North America (Atlantic Compass LR No. 8214176, letter dated 26 April 2004, Ref. NAO/0402535/001/GP) and the *Moku Pahu* system was approved by ABS Americas Division (M/V Moku Pahu ABSID 8208482, letters dated 8 July 2005 REF 503135 and 15 August 2005 REF 510359).

The applied test protocols were communicated with the German Administration (Federal Maritime and Hydrographic Agency of Germany; BSH) and a short description of the various applied methods is included in the next section. During the certification process the whole practical procedure of intake and discharge has been witnessed by the classification society (Lloyds Register) national and international agencies.

NIOZ is currently acting as an official certification organization for land-based testing for the German and UK administration according to approved protocols and final reports will be made public at time of awarding the certificate.

7 Requirements to meet the Regulation-D2

According to D2-Standard of the IMO/MEPC Convention of 2004 (Anonymous, 2005) ships that meet the requirements of the Convention by meeting the ballast water performance standard must discharge:

1) Less than 10 viable organisms per cubic metre greater than or equal to 50 micrometers in minimum dimension;
2) Less than 10 viable organisms less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension and
3) Less than the following concentrations of indicator microbes, as a human health standard:
    1. Toxicogenic *Vibrio chlorerae* (serotypes O1 and O139) with less than 1 colony forming unit (cfu) per 100 milliliters or less than 1 cfu per 1 gramme (wet weight) of zooplankton samples:
    2. *Escherichia coli* less than 250 cfu 100 milliliters;
    3. intestinal Enterococci less than 100 cfu per 100 milliliters.

The present Standard-D2 is defined as a standard for the water characteristics at **discharge**. Furthermore, with exception of some indicator microbes (point 3) organisms < 10 µm are completely excluded. This is certainly an omission since this size class in particular includes numerous phytoplankton species characterized as Harmful Algal Blooms (HABs).
Nevertheless, the standard is clear with respect to the maximum number of organisms remaining present. On the other hand a proper definition of the dimensions of organisms is still subject of (academic) discussion. Moreover, as an operational definition for viable organisms the IMO is using: *organisms and any life stages thereof that are living*, but a more adequate (scientific) definition is: an organism that is able to complete its life-cycle, including reproduction (DNA replication).

In addition to the (basic) requirements for the D2-Standard we have adopted a variety of methods and techniques to determine abundance, sizes and the viability status of different types of organisms. This includes also plankton < 10 µm other than bacteria (i.e. phytoplankton and viruses). Moreover, we extended our research effort to examine not only the fate of the organisms in the large-scale ballast water basins but also took subsamples for incubation under optimal growth conditions in our to study the growth potential of potentially remaining organisms or survival stages such as eggs, cysts or dormant cells over a longer period than the recommended 5 days. These later experiments allowed us also to study potential latent toxicity effects.

7.1 Requirements to meet: guideline G8

Next to the D2-Standard two guidelines were developed by the IMO as a framework for approval of ballast water treatment systems (G8) and approval of the use of active substances in ballast water treatment systems (G9). For land-based testing MEPC 53/Annex 3 (Anonymous, 2005) was compiled of which the most relevant parts will be presented below. These guidelines were generically designed to meet the conditions of a broad range of potentially effective treatment techniques to be tested in typical port and environmental conditions found across the globe. Most test protocols therefore require extensions of the test design to cover the specific aspects of the treatment. The land-based testing serves to determine the biological efficacy of the BWT systems under consideration for Type Approval under more or less controlled and replicable conditions. This is intended to ensure that the efficacy of the equipment is consistent and can be shown repeatedly. The test set-up should therefore be representative of the characteristics of the arrangements used and the type of environment the BWT system was designed for.

One of the main criteria in the G8 test requirements is the salinity range and related to this the differences in Total Suspended Solids (TSS), Particulate Organic Carbon (POC) and Dissolved Organic Carbon (DOC). This resulted in three main categories of test conditions (Table 1).

Table 1: Three different salinity ranges and minimum concentrations of TSS, POC and DOC in the water.

	Salinity			
Parameter	**> 32 PSU**	**3 – 32 PSU**	**< 3 PSU**	**unit**
Total Suspended Solids	**> 1**	**> 50**	**> 50**	**mg/L**
Particulate Organic Carbon	**> 1**	**> 5**	**> 5**	**mg/L**
Dissolved Organic Carbon	**> 1**	**> 5**	**> 5**	**mg/L**

Previous experiments and additional tests, as documented in the application for Basic Approval; (MEPC 58/2/2) showed that the ECOCHLOR®-System was not affected by differences in salinity. The physical step, filtration, will certainly not be affected by salinity but also the reactivity of the active compound (Chlorine-dioxide) will not be altered in the presence of high concentrations of salt. It was for this reason that the Type Approval tests were conducted at the intermediate (3 – 32 PSU) and high salinity (>32 PSU) regions. Moreover, the only difference in composition of the test water between the freshwater and intermediate salinity water is the presence or absence of seasalt. All other minimum requirements for TSS, POC and DOC for these two water types were identical (Table 1).
A further requirement is that the difference between the two salinity regimes should be at least 10 PSU. The test water, originating from the Wadden Sea, and the actual sampling did vary with the tide and as a result salinity was subject to variations. To assure the 10 PSU salinity differences it was decided to have the possibility of adding fresh surface water and upgrade coastal water of the North Sea by enhancing the salinity (brine solution of commercially available sea salt; ca. 18%). As target number the freshwater addition was adjusted to a salinity of ca. 23 PSU for the low salinity regime and ca. 33 for the high salinity regime. In practice ca. 15 % (v/v) of freshwater was added during the low salinity tests and about 4% of brine solution (Instant Ocean®), during the high salinity test runs. In order to compensate the dilution of the TSS by the freshwater some extra sediment (taking from a nearby mudflat) was added as well. These additions were made close at the pump site, to ensure proper mixing, with a constant flow rate and done during filling of the control and the treated ballast tank.

Biology

The guideline G8 also defines criteria for the number and diversity of the organisms to be met during Type Approval testing (Table 2). These criteria should be met for all three salinity regions.

Table 2: Minimal numbers and species diversity required at intake for different size classes and groups of organisms.

Influent test water		
Parameter	**unit**	**Remarks**
organisms ≥ 50 micron	> 10^5/ m^3	at least 5 species from at least 3 different phyla/divisions
10 ≤ organism size ≤ 50 micron	> 10^3/ mL	at least 5 species from at least 3 different phyla/divisions
heterotrophic bacteria	> 10^4/ mL	not further defined

The test water should contain minimum densities of plankton which are typical densities encountered in the Wadden Sea during the annual spring bloom in April/May. With respect to the species diversity, the Wadden Sea is known for its natural richness in organisms and during the test period (April – July) indeed a large diversity in organisms, adults, juveniles, eggs, etc. was encountered.
An important aspect, so far not recognized in the guidelines (G8), when dealing only with natural populations of organisms in the influent of the test water is the natural seasonality of species and blooms. The actual onset of the spring bloom is characterized by a dominance in phytoplankton, but usually lacks high zooplankton abundance. Only at a later stage zooplankton starts to increase in abundance, subsequently due to predation it will diminish the numerical abundance of (smaller size) phytoplankton component.
Furthermore, for the high salinity range, the composition of the organisms in the water resembles that of a typical oceanic environment. This implies an increase of smaller sized cells, down to the micrometer scale, and also a dramatic decline in the number of larger (>10 µm) organisms. So far this shift in community structure has not been accounted for when using natural plankton for testing.

Human pathogens

Table 3: Maximum allowed numbers of 3 groups of indicator microbes in the effluent test water on discharge. cfu: colony forming units

Effluent test water		
Parameter	**unit**	**Remarks**
Toxicogenic *Vibrio chlorerae*	< 1 cfu/100 mL or < 1 cfu/ g wet weight of zooplankton	serotypes O1 and O139
Escherichia coli	<250 cfu/ 100 mL	
intestinal Enterococci	<100 cfu/ 100 mL	

Within the group of prokaryotic microbes only bacteria and more specifically the heterotrophic group (Table 2) has been defined by the standard but for completeness this should include all bacteria and presently also Archeae. While these microbes are part of the natural community in the aquatic environment the indicator microbes (Table 3), i.e. the

human pathogens, are introduced as part of human activity and often associated with sewage discharge. In the present research all microbes have been included as a bulk parameter, the number of heterotrophs as a viable component as well as the viability of the whole microbial community has been determined.
Within the whole microbial community the number of heterotrophic bacteria was determined as well as *E. coli* and total enterococci. The test area of the institute is part of a tidal estuary of the Wadden Sea, which is essentially a pristine environment. Moreover, waste water treatment is highly developed in the Netherlands. Therefore, numbers of these human pathogens during the tests were to be expected to be low for most of the sampling period. On the other hand during the different treatment steps a significant amount of particulate organic material is transferred into dissolved organic carbon (DOC) which acts as an excellent substrate for stimulating growth of (heterotrophic) bacteria.

7.2 Experimental design

A variety of methods were applied to examine the biological efficacy of the ECOCHLOR®-System for the different categories of organisms during the two test series. For detailed description we refer to the outline of the official test protocols for the ECOCHLOR®-System (Anonymous, 2009). Sample handling and volumes were according to the description of the guideline for BWT testing (G8) or described in detail when these guidelines were insufficient or other considerations were taken into account. Subsamples were taken randomly or throughout the whole filling procedure of the tanks. As indicated previously there was great emphasis on analysing the freshly collected samples and having multiple methods to examine numerical abundance and viability. Besides various biological samples there was also a basic set of physical and chemical parameters which were monitored prior, during and after discharge. A short description of each parameter and how it has been analysed is given below.

Physical and chemical properties of test water

Temperature

The water temperature was measured using a calibrated thermometer.

pH

The pH-level is measured using a calibrated pH-meter.

Salinity

For salinity ca. 250 - 500 mL water is sampled and stored at room temperature (glass bottles) until analysis by direct measurement in the laboratory at NIOZ. Salinity of the water was measured after each test cycle using a refractometer (Atago) calibrated against 0 and 33 PSU standard (sea)water. The accuracy of the salinity measurement is 0.5 PSU.

Dissolved Oxygen

The spectrophotometric method of the Winkler method (Winkler, 1888; Pai *et al.*, 1993); Reinthaler, 2006) was used to determine the oxygen concentration in the water. Samples were taken using gastight tubing which was specially fitted to the sampling tubing that was used to sample the ballast simulating tanks. The coded glass bottles are flushed at least three times their volume (ca. 120 mL) with water.
The sample bottles were stored in a dark container filled with water of the same temperature as the samples until further analysis at the laboratory. In the laboratory 1 mL H_2SO_4 is added prior to measuring the OD at 456 nm with a Hitachi U-3010

Spectrophotometer. The oxygen concentration was calculated using standards and expressed as μM O_2/L (or mg O_2/L; = μM O_2 * 0.032)

DOC

The concentration of dissolved organic carbon (DOC) was measured according to Reintaler & Herndl (Reinthaler and Herndl, 2005). Samples for DOC (15 mL) were filtered through GF/C filters and sealed in pre-combusted glass ampoules after adding 50 μl of phosphoric acid (H_3PO_4). Sealed ampoules are stored at 4 °C. The DOC concentration was determined in the laboratory by the high temperature combustion method using a Shimadzu TOC-5000 analyzer. Standards were prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is < 3 %.

TSS / POC (total suspended solids and particulate organic Carbon)

For TSS/POC pre-weighted glass fibre filters (GF/C) are used. Each filter was coded and stored in a clean Petri dish. The filtered volume was dependent on the particle load and concentration and type of organisms present in the water. The higher the total particle load in the sample, the smaller was the volume that could be filtered before the filter clogs. Practical volumes were between 100 and 1000 mL per sample.
After filtration the filter was rinsed with fresh water (MiliQ) to remove sea salt. Filters were dried overnight at 60 °C and allowed to cool in a vacuum exicator before weighing. The total amount of suspended solids was calculated from the weight increase of the filter and averaged for the three replicates (mg/L).
Next, the filter is combusted at 500°C (overnight) and allowed to cool in a vacuum exicator and weighted again. The POC was calculated from the weight decrease between this measurement and the TSS weight.

Analytical determination of chlorine-dioxide
During each test cycle, the concentration of the active compound (chlorine dioxide, ClO_2) was measured as soon as possible after filling of the treated ballast water tank and also at regular intervals until the detection limit of the applied method was reached.

Chemistries that have been tested and verified by the United States Environmental Protection Agency (USEPA) are used to analyze the chlorine dioxide treated ballast water produced by the Ecochlor® BWTS. Chlorine dioxide residuals are measured using the lissamine green B (LGB) chemistry (USEPA Method 327).

Figure 4: Example of sample collection point at tank 3.

Chlorine dioxide measurements are performed using the Palin-test 1000 Chlordiox-Duo Photometer with prepackaged reagent kits to measure chlorine dioxide in the 0.1 to 20 mg/L concentration range. The LGB detection chemistry is highly selective for chlorine dioxide in the presence of chlorite ion. The prepared reagents establish the proper pH to

minimize any potential reaction with chlorite ion. The reagent kit also includes a chemical mask to react with chlorine if it is present in the sample. Under the measurement conditions, studies indicate that the seawater matrix does not create interference issues that might lead to inaccurate measurements. The recommended operating range is 0 – 40 °C, and the test reagents are calibrated for use at 15 – 25 °C.

The sensitivity of the Palin-test LGB analytical method provides field verification of the expected decay of chlorine dioxide in treated ballast water at levels below environmentally relevant concentrations.

Biology

The majority, but not the entire large size fraction (> 50 µm) consists of zooplankton, while the majority of the small size (10 - 50 µm) fraction consists of phytoplankton. Organisms > 50 µm are retained as recommended in the G8 guidelines.
Samples for the 10 - 50 µm fraction were collected from the effluent of the Hydrobios net. These samples were then filtered over a 10 µm sieve and fixated.
A second set of samples for this size class was taken and not separated from the organisms < 10 µm in order to include the fate of the smaller sized (phyto)plankton community as well and to avoid further damage of the plankton. The results of these samples were compared to the ones from the double filtered samples to evaluate the loss of organisms caused by processing the samples.

Sample sizes

During the land-based tests containers from 1 to 1000 L were used for sampling and/or storage. Samples were taken continuously and evenly during the whole process of filling or emptying the ballast water tanks. These containers were thoroughly rinsed or heat-treated prior to use. Samples for the human pathogens were taken in sterile (bar-coded) bottles provided by the bacterial test laboratory.

Figure 5: 1000 L container with a 50 µm Hydrobios sampling net

Organisms > 50 µm

The samples are pre-concentrated over a Hydrobios 50 µm net resulting in an end volume of approximately 100 to 200 mL. The samples were transferred to the lab directly after sampling and Neutral Red was added in a ratio that yields an end concentration of 1:50,000. Staining time is 2+ hours. Neutral Red stains living organisms (Crippen, 1974; Fleming and Coughlan, 1978) distinctively and quite rapidly (less than one hour, figure 6). Therefore the

viability assessment remains unaffected by the possible death of organisms during the staining or during sample analysis.
It is assumed that dead but physically intact organisms will also be found. Consequently a detailed inspection of each intact individual is needed to assess viability. This includes the staining as well as the detection of internal (heart, gills) movement. Organisms which were not intact are assumed to be dead.

Neutral Red is a reliable staining method for all major groups of organisms but inconsistent staining was found for bivalves. For this latter group movement (including internal such as heart and gills for juvenile mussels) has to be used obligatorily to determine viability. This is dependent on the expertise of the person analyzing the samples. Therefore the same person analyzed all samples.

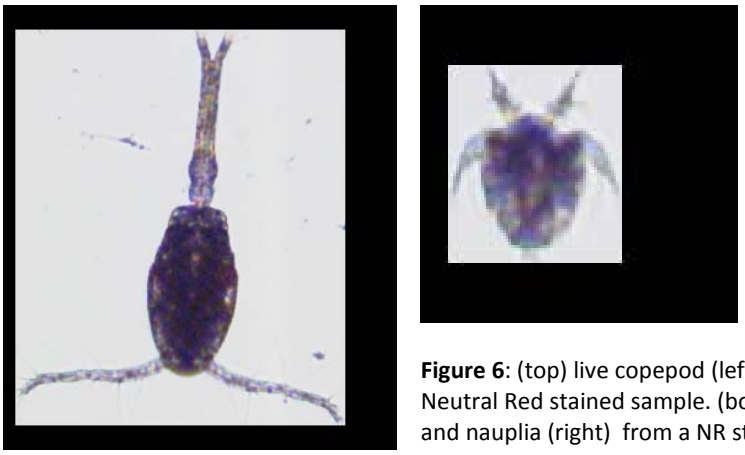
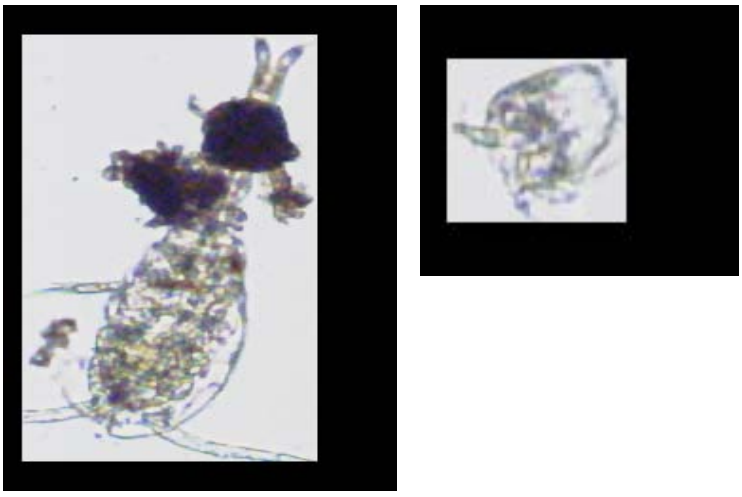


Figure 6: (top) live copepod (left) and nauplia (right) from a Neutral Red stained sample. (bottom) dead copepod (left) and nauplia (right) from a NR stained sample.



The samples are analyzed manually using a binocular with a 20x magnification for counting and up to 50x for species identification and measurements when necessary. For inter comparison a subset of samples was also analyzed using a semi-automated tool (FlowCam, Fluid Imaging Technologies; (Anonymous, 2001). Organisms need to be counted according to their size. Here organisms of 50 µm in minimum dimension are relevant. Several tests have shown that a single size bar is not efficient as viable organisms move in the counting chamber. Better results are achieved when the entire field of view is equipped with a size grid. The minimum dimension to measure will be adjusted to the specific organism groups.

Organisms < 50 µm

Samples for visual inspection of species number and diversity were pre-concentrated using a sieve made of a Hydrobios 10 µm mesh net using the 50 µm prefiltered sample (effective size range is 10 – 50 µm). The retained organisms were flushed into 50 mL Greiner tubes using filtered seawater and fixed with Lugols solution. Sample analysis was conducted by microscopic count with an inverted microscope at 200x magnification (method by Utermöhl). Since the Utermöhl is not suitable to asses viability counting was restricted to the structural integrity of organisms and therefore the presence of intact cells (Paerl, 1978). This method works for both zoo- and phytoplankton.
This size fraction was also covered by flow cytometry on basis of a single cell measurement (Veldhuis and Kraay, 2000) and PAM fluorometry, as a bulk parameter (Schreiber *et al.*, 1993), using the intact and undisturbed samples. Besides numbers and sizes these two methods can be used to assess the cell viability (Veldhuis *et al.*, 2001; Veldhuis *et al.*, 2006) or in case of the PAM fluorometry also the photosynthetic efficiency of the phytoplankton.

Flow cytometry: For total organisms counts 3 mL of unfiltered sample water (reference and treated, each in triplicate) were analyzed using a calibrated flow cytometer. This yields the total number of particles (dead and live organisms as well as detritus) as well as their size range and the presence or absence of chlorophyll. For the counts exactly 1 mL was analyzed. The size of the plankton was determined by comparison to standardized beads (10 and 50 µm). These beads were also used as standards to calibrate the performance of the flow cytometer.
For organism viability testing, on the level of the individual cell, SYTOX Green was added to 1 mL of sample water (control and treated, each in triplicate). After 15 minutes samples were analyzed using the flow cytometer for the presence of dead and/or live organisms (cf. Veldhuis et al, 2001).

Figure 7: (A) Epifluorescence microscopic picture of a live phytoplankton cell. The red signal is due to the presence of chlorophyll and (B) a dead phytoplankton cell with a yellow/green fluorescence of the nucleus after staining with SYTOX Green.

PAM fluorometry: The photochemical efficiency of photosystem II of phytoplankton (providing an estimate of the general health of the algae) can be addressed using Pulse-Amplitude Modulated fluorometer (PAM-fluorometry) WALTZ- water PAM (Schreiber *et al.*, 1993). For this 3 mL of unfiltered sample water (control and treated, each in triplicate) are filled into a glass cuvette and analysed using the Pulse-Amplitude Modulated fluorometer. The instrument was calibrated against filtered seawater and a healthy fast-growing population of phytoplankton.

Next to cell specific analysis Plant-pigments and chlorophyll *a* were determined to assess the fate of the whole phytoplankton community (Jeffrey *et al.*, 1997; Kraay *et al.*, 1992). For

this purpose water samples of 0.2 to 1.5 L (GF/C filters) were taken. The samples were frozen until further analysis.

The system used is a Dionex HPLC system equipped with a C18 separation column. The different algal pigments can be separated according to their polarity. The following solvents are used as elutes in the HPLC gradient: A 0.5M ammoniumacetate in methanol and water (85:15), B acetonitril and water (90:10) and C ethylacetate 100%.

Bacteria

The classical method for counting bacteria in many applications is based on plating on selective media. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol and Del-Giorgio, 2000). As a result total bacteria were now determined by flow cytometry, using DNA-specific stains to get a more accurate bacteria number. In addition samples were taken at discharge for specific human pathogens and heterotrophic bacteria using a plate method.
A 1.5 mL water sample was taken and pipetted in a Cryovial (in triplicate) and formaldehyde was added as a preservative. Samples were frozen and stored at -50 ºC until further analysis.
Upon analysis the sample is allowed to thaw completely. A subsample of 100 µl is taken, diluted with a TE-buffer, and the nucleic acid dye PicoGreen (MP) was added. Within 5 to 15 minutes after the addition of the stain the sample is analyzed using a flow cytometer (cf. (Gasol *et al.*, 2000; Veldhuis *et al.*, 1997). A known bacterial standard is used for calibration and counting.
The number of total heterotrophic bacteria was determined using a plate method as the number of colony forming units (cfu's) after incubation of the water at intake and discharge according to an international standard (NEN-EN-ISO 6222:1999).

Human pathogens

The samples for microbiological analysis are taken in special bottles of 600 mL and send to a special laboratory for further analysis. This laboratory was "Vitens laboratory bv" at Leeuwarden (accreditation certificate: NEN/ISO/IEC 17025; lab. no. L043). All analysis' are carried out according to NEN-EN-ISO standards.
These samples are sent to the laboratory immediately after sampling using a cooled transport container (4 ºC). The analysis is carried out according NEN-EN-ISO 7899-2 for intestinal enterococci and NEN-EN-ISO 9308-1 for *E. coli* and related bacteria of the coli group as adopted for surface and waste water analysis in the Netherlands.

8 Results

The present section is a compilation of all relevant information needed for Type Approval Certification tests according to the Guidelines (G8), but also includes some relevant results of experiments conducted to assess the environmental effect of the active substance and/or its by-products in the environment upon discharge. It should however, be noted that detailed studies regarding potential toxicity according to standard procedures have also been carried out. These results were submitted to IMO/GESAMP-BWWG. Data are presented as averages or ranges separated for the two salinity regimes tested. In Annex 1 a detailed species list of organisms observed is presented.

Figure 8: overview of the ECOCHLOR®-System (filter and container) in the NIOZ harbour (top) and detail of the internal disinfection unit (bottom).

The tests were carried out at two different salinity regimes (Tables 4, 5 and 6) hereafter referred to as low and high salinity test series.

8.1 Physical and chemical parameters

Table 4: Average salinity and temperature of water at intake during the first low salinity tests of control and treated tanks for test runs I – III.

test run	salinity [PSU]	s.d.	temperature [°C]
I –III	22.1	0.92	8.5 - 10.4

To the low salinity test cycles brackish water from the NIOZ harbour was collected during low tide and fresh water, taken from Lake Ijssel was added to a maximum of 15% (v/v).

Table 5: Average salinity and temperature of water at intake during the high salinity tests of control and treated tanks for test runs VI – X.

test run	salinity [PSU]	s.d.	temperature [°C]
VI - X	31.9	1.3	14.3 - 17.5

To the high salinity test cycles coastal water from the North Sea was collected during high tide and a brine solution made from natural sea salt was added to a maximum of 4 % (v/v).

During the first three test runs it became clear that with respect of the organism > 50 µm the ECOCHLOR®-System was not in compliance with the Standard-D2. The test runs at low salinity were repeated after the high salinity test series (Table 6). All data therefore exclude the results of the first test-runs (table 4) unless indicated otherwise.

Table 6: Average salinity and temperature of water at intake during the second low salinity tests of control and treated tanks for test runs XI – XVI.

test run	salinity [PSU]	s.d.	temperature [°C]
XI - XVI	23.1	0.65	16.7 - 18.8

In regards to salinity, the difference between both the high and low salinity regimes is slightly less than 10 PSU. However, given the standard variation and the fact that tidal effects and prevailing winds occasionally disturbed the ideal situation, the minor deviation is considered to be of minor importance. Moreover, efficacy of the Ecochlor-BWT-system is not affected by changes in the salinity.

Low salinity

A summary of the results of the basic parameters (oxygen concentration, pH, TSS, POC and DOC) is presented in Table 7 for the reference and treated water sample at intake and discharge.

Table 7: Oxygen, pH, TSS, POC and DOC concentrations of test series run at low salinity (6 test runs in total; XI-XVI) at intake and discharge;
[1]: range of values; the oxygen concentrations are corrected for the interference of the active substances or derivates of it.

Parameter	Intake	s.d.	Discharge (day 5)	s.d.	unit
O_2 reference[1]	5.7-8.5		3.5-5.9		mg/L
O_2 treated[1]	10.6-13.3		9.9-12.0		mg/L
pH reference	7.95	0.09	n.d		
pH-treated	6.91	-	6.92	-	
TSS-reference	75.3	23.6	14.0	9.8	mg/L
TSS-treated	19.3	9.50	13.0	2.1	mg/L
POC -reference	11.6	1.50	7.60	3.0	mg/L
POC-treated	9.0	3.90	6.60	1.4	mg/L
DOC -reference	3.2	0.47	2.8	0.37	mg-C/L
DOC-treated	3.4	0.36	3.5	0.43	mg-C/L

Table 7 clearly demonstrates that as far as the basic parameters are concerned the values were in accordance with the criteria as indicated in the guidelines (G8, Table 1). As far as the oxygen values of the reference tanks are concerned values at intake were closely corresponding to a saturation values (100%) for the given salinity and temperature with exception of 1 test run (69% saturation level). Since the water temperature increased steadily during the test series values are presented as a range and not averaged. At discharge on day 5, the oxygen concentration in the reference tank declined by ca. 33% compared to intake values. In contrast, oxygen concentration in the treated tank tended to be higher, by as much as 60% as compared to the reference tank. This apparent super-saturation is partly an artefact caused by the active substance chlorine-dioxide interfering with the Winkler method for O_2 measurements. Separate tests showed that for the applied concentrations of chlorine-dioxide added at least half of the observed increase in the oxygen concentration could be accounted for. After the required incubation period of 5 days the oxygen concentration dropped only slightly (ca. 10%).

The total suspended solids (TSS) and particulate organic carbon (POC) concentrations were sufficiently high at intake but these values varied considerably among the different test runs. At discharge the sediment concentration declined significantly, mainly because of sedimentation in the tank during the holding period of 5 days. Compared to the reference tank, the applied filter of the Ecochlor system effectively reduced the amount of TSS at intake and partly also the POC. Most of this material remained in suspension. At discharge on day 5, both TSS and POC were reduced in the treated tank, but the reduction was far less than was measured in the reference tank.

High salinity

Table 8: Oxygen, pH, TSS, POC and DOC concentrations of test series run at high salinity (5 test runs in total; VI-X) at intake and discharge;
[1]: range of values; the oxygen concentrations are corrected for the interference of the active substances or derivates of it.

Parameter	Intake	s.d.	Discharge (day 5)	s.d.	unit
O_2 reference[1]	4.7-9.2		2.6-6.1		mg/L
O_2 treated[1]	8.8-12.0		8.0-10.9		mg/L
pH reference	8.07	0.05	n.d.		
pH-treated	6.81	-	6.89		
TSS-reference	33.9	37.10	9.70	3.10	mg/L
TSS-treated	15.6	3.60	9.60	1.30	mg/L
POC -reference	10.2	5.30	4.80	1.30	mg/L
POC-treated	7.8	2.10	4.80	0.50	mg/L
DOC -reference	4.0	1.02	3.3	0.51	mg-C/L
DOC-treated	4.3	0.76	4.3	0.93	mg-C/L

The results of the basic parameters at the high salinity range were, with a few exceptions, on the whole the same as for the low salinity range (Table 8). At intake the ambient water was, with exception of a single test run, saturated with oxygen and at discharge oxygen concentrations in the both control and treated tanks declined only slightly. Oxygen values were still far from depleted. Partly because of the increase in seawater from the open North Sea sediment, TSS values were lower but POC remained high, as a result of the high content of organisms, as compared to the test series conducted at low salinity. Mainly due to sedimentation the residual TSS load and POC after the 5 day holding period was reduced to ca. 10 mg/L and 4.8 mg/L, respectively.

8.2 Biology

Organisms > 50 µm

For the land based tests natural plankton was used and the required diversity of organisms (5 species of at least 3 different phyla) was easily fulfilled for all tests (Table 8). On average at least 10 different species of 4 to 5 different phyla were present in each sample (full details on species present see Annex 1).
Regarding the required minimal numbers of organisms per volume (Table 2), the values for the low salinity series (both test series runs 1 - 3 and 11 - 16) were, with exception of run 11/12, well above the minimum requirements. In some cases the numbers in the >50 µm size fraction were 48 times higher (test run II) than the minimum required number of 100,000 m^{-3}. At the high salinity range the water at intake contained in three cases insufficient numbers (test runs 8/9 and 10) although on average the numbers did meet the minimum requirement. As explained earlier this is mainly due to the fact that natural seawater was used without any addition of organisms and that on average numbers of larger sized organisms is known to be much lower in high saline water than brackish water.
During the first three test runs measurements indicated that the numbers of the organisms at discharge exceeded the maximum allowed number of 10 organisms m^{-3}. Close observation showed that in particular the hard shelled plankton (cypris larvae of barnacles) was only partly affected by the chemical treatment and a significant number were still alive at discharge. Barnacles are found world-wide in costal habitats. They form a major part of

the biomass on many hard-substrates in coastal areas. Most barnacles undergo the stage of cypris larvae during their life cycle. In this life stage the larvae posses a hard shell which they can close entirely. They do not take up food during this stage and for some species have been shown to survive up to 20 days as cypris. A number of barnacle species has already been identified as invading new habitats using ships as vectors. This group therefore is important in the scope of ballast water treatment and any system must be able to successfully deal with them.
This resulted in a modification of the treatment system, which added a self-cleaning filter (BSFc, 40 µm filter) phase prior to treatment with chlorine dioxide.

As a result the whole set of test runs at the lower salinity range were repeated (test runs 11 – 16). The installation of the filter turned out to be an effective way of eliminating the larger fraction of organisms. For both salinity regimes the remaining number of organisms in the size class > 50 µm were at least one order of magnitude lower than the Standard-D2. Moreover, at the low salinity range no living organisms were observed in the 6 replicate tests.

As mentioned above in Section 6.4, a 4 mg/L treatment of chlorine dioxide was applied during test run 6. This was done to determine if the addition of the filtration phase would allow the chlorine dioxide dosage to be reduced. The results for test run 6 clearly meet the IMO D-2 standard. However, the remaining treatment cycles were performed with 5 mg/L chlorine dioxide treatment to demonstrate that the more stringent discharge standards being proposed in the United States can be achieved with the Ecochlor System.

As opposed to the treated tank, numbers of organisms in the reference tank were still significantly above the G8 requirement during discharge and visual inspection and viability measurement indicated that these organisms were both intact and viable.

Table 8: Number of organisms > 50 µm at intake and on discharge at day 5 of the reference and treated tank. For test run XI, XIII and XV the same control was used, since this was a combined test run. All numbers are presented per m^3. nd: no data

total plankton > 50 µm			numbers/m^3
low salinity	Intake	Reference	Treated
test run	C-T0	C-T5	E-T5
I	1.24E+6	10.8E+3	3.0
II	4.83E+6	0.62E+3	81.0
III	1.01E+6	2.18E+3	25.3
IV	3.12E+6	2.18E+3	nd
V			nd
average	2.55E+6	3.95E+3	36.4
s.d.	1.79E+6	4.65E+3	40.2

Table 8: continued

total plankton > 50 µm			numbers/m^3
high salinity	Intake	Reference	Treated
test run	C-T0	C-T5	E-T5
VI	4.11E+5	1.31E+4	3.7
VII	2.75E+5	1.67E+4	0.0
VIII	8.57E+4	0.25E+4	0.3
IX	5.15E+4	1.73E+4	0.3
X	6.85E+4	2.76E+4	0.0
average	1.78E+5	1.54E+4	0.9
s.d.	1.58E+5	9.05E+3	1.6

total plankton > 50 µm			numbers/m^3
low salinity	Intake	Reference	Treated
test run	C-T0	C-T5	E-T5
XI	3.57E+4	8.38E+3	0.0
XII			0.0
XIII	3.61E+5	8.57E+3	0.0
XIV			0.0
XV	1.32E+5	2.70E+3	0.0
XVI			0.0
average	1.76E+5	6.55E+3	0.0
s.d.	1.67E+5	3.34E+3	0.0

Organisms 10 – 50 µm

This size class was dominated by phytoplankton, although heterotrophic organisms like ciliates and flagellates were occasionally present in high numbers at intake. For that reason the flow cytometric analysis included chlorophyll *a* fluorescence as an extra selection parameter. Moreover, the photosynthetic efficiency of the phytoplankton community was measured, as a tool to determine the efficacy of the treatment on this plankton group more specifically. Finally, a 10 L subsample of both the reference and treated water was collected at intake and incubated under optimal growth conditions in order to determine potential growth of cells which have survived the treatment or hatching of resting stages/spores. Results of plankton numbers in the 10 – 50 µm of both reference and treated incubation are included in the table for comparison with the actual measurement of the tanks (Table 9).

Table 9 shows that the phytoplankton community in the reference tank responded as was observed in earlier experiments. Pumping the water into the tanks and the prolonged dark period reduced the total number of plankton to 10% of the value at intake. However, the growth potential of this phytoplankton was very high as can be derived from the increase in the numerical abundance when a subsample of the water at intake was incubated under optimal growth conditions (C-Inc-T5).
In contrast in the treated tank the number of intact cells immediately after intake (E-T0) declined to an extreme low value. At this stage the remaining cells do meet the requirements as defined in the Standard-D2. The incubation period of 5 days resulted in a

further reduction of the cell numbers and on average the remaining number of viable cells was < 0.1 per mL. Testing for regrowth also indicated that the treatment was very effective and as no viable cells were observed in the incubated samples (E-inc-T5).
As expected and corroborating with the results for the larger organisms no effect was observed of the difference in salinity regime.

Table 9: Total plankton number of plankton in the 10 – 50 µm size range at intake, reference (C) and treated tank (E), reference and treated incubated samples (Inc.) at day 5 (T5) during discharge. Runs VI to X at high salinity range and XI to XVI at low salinity range. Test runs XI, XIII and XV share the same reference since this was a combined test run. All numbers are presented per mL as total counts or of viable cells (*).

10- 50 µm cell numbers cells/ml							Ecochlor
high salinity	Intake	Reference		Treated	total	viable	
test run	C-T0	C-T5	C-inc-T5	E-T0	E-T5	E-T5*	E-inc-T5
VI	1470	121	9391	4.4	3.7	<0.1	1.5
VII	1345	149	6007	0.0	0.0	<0.1	0.0
VIII	3207	149	7774	0.0	0.0	<0.1	0.0
IX	730	130	5754	8.1	0.0	<0.1	0.0
X	1385	151	5941	0.7	0.0	<0.1	0.7
average	1628	140	6973	2.7	0.7	<0.1	0.4
Low salinity							
XI	1746	175	3766	5.9	0.0	<0.1	0.0
XII				0.0	0.0	<0.1	0.0
XIII	1512	130	7776	0.7	0.7	<0.1	0.0
XIV				2.2	0.0	<0.1	0.0
XV	719	165	38544	2.2	0.0	<0.1	0.0
XVI				0.0	0.0	<0.1	0.0
average	1326	157	16695	1.9	0.1	<0.1	0.0

Organisms <10 µm

In order to have a more complete insight of the fate of all organisms, i.e. also fraction the planktonic fraction < 10 µm in diameter, a group of phytoplankton in the size range of ca. 6 µm was monitored in the control and treated water (Table 10). Although no clear criteria are, yet, defined for this size class it was clear that the Ecochlor®-System was also effective in reducing organisms in this size range. Flow cytometric analysis showed the presence of a extreme low number of intact cells in the treated tanks immediately after intake. After 5 days of holding the numbers declined even further and in terms of viable cells the number was < 0.1 per mL at discharge. Incubation of chlorine dioxide treated water under ideal growth conditions did not alter the general observation since no regrowth of phytoplankton was observed (E-inc-T5).

Table 10: Phytoplankton number of a dominant phytoplankton species present (*Phaeocystis globosa*; ca. 6 µm in size) at intake, reference and treated tank, reference and treated incubated samples (Inc.) at day 5 during discharge. Runs VI to X were test runs at high salinity range and XI to XVI at low salinity range. Test runs XI, XIII and XV share the same reference since this was a combined test run. All numbers are presented per mL as phytoplankton counts or of viable cells (*).

Phaeocystis ~ 6 µm cells/ml							Ecochlor
high salinity	Intake	Reference			Treated	total	viable
test run	C-T0	C-T5	C-inc-T5	E-T0	E-T5	E-T5*	E-inc-T5
VI	4556	632	9458	0	3.0	<0.1	0.7
VII	1795	204	4364	0	0	<0.1	0
VIII	2106	204	6650	2.2	2.2	<0.1	2.2
IX	1004	236	21473	0.7	0	<0.1	0
X	1857	173	14440	4.4	0	<0.1	1.5
average	2264	290	11277	1.5	1.0	<0.1	0.9
low salinity							
XI	8460	393	8574	76.2	0.0	<0.1	0.0
XII				4.4	0.0	<0.1	0.7
XIII	15096	1195	27619	5.2	1.5	<0.1	0.0
XIV				9.6	0.0	<0.1	1.5
XV	3278	548	36220	3.0	6.7	<0.1	0.7
XVI				46.6	4.4	<0.1	0.7
average	8945	712	24138	24.2	1.6	<0.1	0.6

Photosynthetic efficiency

Another approach to gain insight in the physiological condition and therefore the growth response of the phytoplankton community is by measuring the photosynthetic efficiency (Fv/Fm) of the cells. This was done for the whole community, but is also possible for each of the different size fractions. For a clear understanding it must be noted that values of Fv/Fm > 0.4 are indicative of a healthy phytoplankton population; an Fv/Fm < 0.4 indicates that the phytoplankton community is experiencing severe stress and a value < 0.1 is typically observed in decaying phytoplankton populations.
Table 11 shows that, with one exception (test run 7) during intake the whole phytoplankton community in the water was in a physiologically healthy condition, i.e. containing mostly photosynthetic active and therefore viable phytoplankton cells. Prolonged incubation of the control in the dark (5 days of dark is a considerable stress condition for algae) resulted in a considerable reduction of the photosynthetic efficiency to values indicative of severe stress and occasionally values were measured typical of a decaying population.
In contrast the photosynthetic efficiency of a subset of phytoplankton of the control taken at intake and incubated under optimal growth conditions was typical for a healthy and viable phytoplankton community (C-inc-T5). This is not surprising since phytoplankton also increased in numerical abundance in these subsamples (Tables 9 and 10).
After passing the treatment system no distinct phytoplankton cells were observed and therefore Fv/Fm was typical of a decaying algal community. Prolonged storage in ballast tanks, but also in the incubated sample under optimal growth conditions, showed that there was no recovery of the phytoplankton population.

Table 11: Photosynthetic efficiency (Fv/Fm) of the whole phytoplankton community (2 - > 50 µm size range) at intake, in the reference and treated tanks at discharge and in the incubated samples taken from the reference and treated tank after days of incubation. Runs VI to X were test runs at high salinity range and XI to XVI at low salinity range. Test runs XI, XIII and XV share the same reference since this was a combined test run.

high salinity	Intake	Reference		Treated		
F_v/F_m	C-T0	C-T5	C-inc-T5	E-T0	E-T5	E-inc-T5
VI	0.59	0.13	0.63	0.00	0.00	0.00
VII	0.44	0.11	0.65	0.00	0.00	0.00
VIII	0.67	0.16	0.60	0.00	0.00	0.00
IX	0.62	0.28	0.72	0.00	0.00	0.00
X	0.66	0.13	0.64	0.00	0.00	0.00
average	0.60	0.16	0.65	0.00	0.00	0.00
s.d.	0.09	0.07	0.04	0.00	0.00	0.00
low salinity		Reference		Treated		
XI	0.56	0.10	0.73	0.13	0.00	0.00
XII				0.00	0.00	0.00
XIII	0.65	0.42	0.72	0.00	0.00	0.00
XIV				0.00	0.00	0.00
XV	0.51	0.24	0.63	0.02	0.00	0.00
XVI				0.00	0.00	0.00
average	0.57	0.25	0.69	0.03	0.00	0.00
s.d.	0.07	0.16	0.05	0.05	0.00	0.00

Bacteria

For the microbial community the presence/absence of two types of human pathogens (*E. coli* and enterococci) was monitored prior to and after treatment, while the response of the whole microbial community was also assessed.
Table 12 shows that even during intake (C-T0) the number of both target microorganisms was well below the standard as indicated in the Standard-D2. In five test runs the numbers of human pathogens were above the detection limit for one or both target microbes (test runs 7, 8, 9, 11, and 15). The reason for this is that the NIOZ harbour is located in a pristine environment with little or no urban activity. Subsequently, also the number of human pathogens in the reference and treated tanks were below detection limit during discharge.

Table 12: Counts of the human pathogens *E. coli* and total enterococci (as cfu's) at intake and during discharge of the reference and treated tank. nd; no data. Test runs XI, XIII and XV share the same reference since this was a combined test run.

human pathogens Intake			Reference		Treated	
counts/mL	C-T0	C-T0	C-T5	C-T5	E-T5	E-T5
high salinity	*E.coli*	Enterococci	*E.coli*	Enterococci	*E.coli*	Enterococci
VI	<0.1	<1	<0.1	<1	<0.1	<1
VII	nd	1		<1		<1
VIII	<0.1	17	<0.1	<1	<0.1	<1
IX	<0.1	7	<0.1	<1	<0.1	<1
X	<0.1	<1	<0.1	1	<0.1	<1
average	<0.1	5.0	<0.1	<1	<0.1	<1
low salinity						
XI	<0.1	1	<0.1	<1	<0.1	<1
XII					<0.1	<1
XIII	<0.1	<1	<0.1	<1	<0.1	<1
XIV					<0.1	<1
XV	1.4	27	<0.1	1	<0.1	<1
XVI					<0.1	<1
average	0.5	9.3	<0.1	<1	<0.1	<1

In contrast to the near absence of human pathogens, the typical marine microbial community was abundantly present. The total bacterial community is well studied in the Wadden Sea, using a method based on staining the nucleic acid of the cells for many years. The currently observed numbers ranging from 1.25 to 9.02 10^6 per mL (variation of factor 7.2) are numbers typically observed in spring and early summer (Table 13). Of this total bacterial population only a very small fraction could be identified as heterotrophic ones using the plate assay method (<10 to 50 per mL). Therefore, if only plating would be used as criteria for heterotrophic bacteria number this would result in a severe underestimation of the actual bacteria numbers. After 5 days of incubation total bacteria numbers on average declined in the control tank but there was considerable variation between the different test runs (C-T5).

Bacteria numbers in the incubated water of the reference tanks did not follow the pattern observed for the treated tank samples. In the high salinity test series there was on average a slight increase in bacteria numbers after 5 days whereas during the low salinity test series average number was comparable to the values observed for the tank.

In the treated tanks and in the incubation bottles of the same water the total bacteria numbers at discharge varied from the original numbers during intake in an inconsistent manner. During the high salinity test run there was in nearly all experiments a considerable increase in bacterial number (on average 2 fold increase relative to intake). In contrast to the low salinity test there was considerable variation between the different test runs but on average numbers in the treated water were lower than in the control. The apparent increase in bacterial numbers at the high salinity test runs is most likely due the presence of chloroplasts and mitochondria which are liberated during the disintegration of the larger

organisms. The size and the DNA content of these cell constituents are matching that of free living marine bacteria.
The treatment with chlorine dioxide was acting as a disinfection agent since bacteria numbers in both the tank and in the incubation bottle showed only moderate changes during the 5 days of incubation.
This disinfection also resulted in a decline in the number of colony-forming heterotrophs, measured as cfu's. However, in two tests the chemical treatment could not prevent bacterial activity resulting in high increase in heterotrophic bacteria thriving on the high dissolved organic carbon load of the water.

Table 13: Total bacteria number (total bact.) and colony forming heterotrophs (colony = cfu numbers per mL) at intake (C-T0) and during discharge of the reference (C-T5) and treated (E-T5) tank as well as the total bacteria numbers in the incubated water samples (inc.). Test runs XI, XIII and XV share the same reference since this was a combined test run. * range of values

bacteria	Intake		Reference			Treated			
	C-T0	C-T0	C-T5	C-inc-T5	C-T5	E-T0	E-T5	E-inc-T5	E-T5
high salinity	total bact.	colony	total bact.	total bact.	colony	total bact.	total bact.	total bact.	colony
VI	5.42E+6	20	0.94E+6	6.11E+6	10	7.94E+6	4.80E+5	3.28E+6	1700
VII	1.25E+6	50	5.37E+6	8.17E+6	20	9.54E+6	6.15E+6	9.74E+6	<10
VIII	4.64E+6	20	1.68E+6	5.15E+6	30	3.03E+6	2.86E+6	5.87E+6	40
IX	3.21E+6	30	0.33E+6	3.00E+6	<10	1.13E+7	8.24E+6	1.15E+7	<10
X	4.45E+6	10	2.40E+6	6.26E+6	60	7.11E+6	6.77E+6	7.65E+6	<10
average	3.79E+6	26	2.15E+6	5.74E+6	26	7.78E+6	4.90E+6	7.61E+6	<10-1700*
low	salinity								
XI	3.66E+6	45	1.89E+6	6.24E+5	700	4.50E+6	3.69E+6	6.29E+6	>1000
XII						3.37E+6	3.14E+6	3.74E+6	<10
XIII	6.72E+6	<10	2.90E+6	3.56E+6	20	7.95E+6	7.86E+6	6.83E+6	<10
XIV					<10	5.30E+6	5.07E+6	5.39E+6	<10
XV	9.02E+6	20	0.86E+6	2.52E+6		8.58E+6	6.13E+6	8.82E+5	<10
XVI						3.10E+6	2.54E+6	3.72E+6	<10
average	6.47E+6	25	1.89E+6	2.23E+6	243	5.47E+6	4.74E+6	4.47E+6	<10->1000*

9 Environmental acceptability

Ballast water treatment systems applying active substances, and also systems not using active substances according to the latest version of the guidelines G8 (MEPC174.58), should demonstrate that the treated water upon discharge is not harmful to the environment and aquatic organisms.

Although not obligatory for the present system a series of studies were carried out to examine long-term (20 days) regrowth and vitality experiments. In total 3 long-term incubation experiments (2 at low salinity and 1 at high salinity range) were conducted with treated water which was in collected in a clean container (10 L) and incubated under optimal growth conditions in a climate room. To stimulate regrowth of planktonic organism extra nutrients were added (nitrate; 30 µM, phosphate; 2 µM, silicate; 20 µM). In particular at the peak of the spring bloom and thereafter the ambient nutrient concentration (mainly silicate and phosphate) can be extremely low, which may prevent growth of phytoplankton and also bacteria.

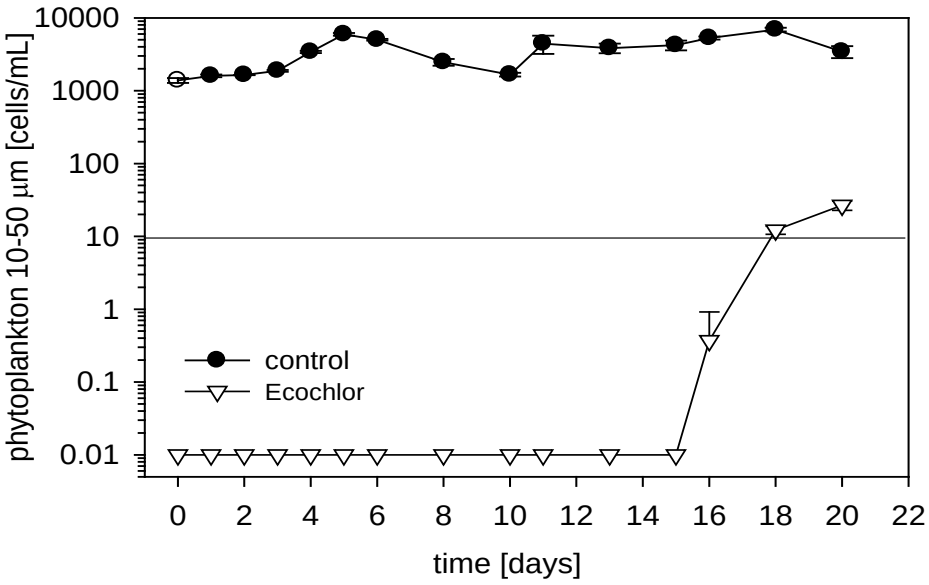


Figure 9: time course of phytoplankton numbers (10 – 50 µm size range) during a 20 day incubation period, control and treated water (Ecochlor). Test run X, values are mean of three replicates.

Figure 9 shows that regrowth of phytoplankton, in this case only the fraction of 10-50 µm is shown was found after 15 days of incubation. In the two other studies no phytoplankton regrowth was observed, of any size class, during the whole incubation period of 20 days. These results indicate that the treatment was very effective in killing or deactivating phytoplankton but also in preventing hatching of resting stages or cysts for a considerable period.

Based on the first two similar types of experiments it was evident that as far as phytoplankton was concerned the treated water was free of **viable** phytoplankton cells for a considerable period of time. The virtual absence of organisms for a prolonged period would suggest that the treated water would be **non-vital** or '**dead-water**'. Specific toxicity studies and the presence of undesirable chemical end- or by-products, associated with the present land-based are documented in a separate report (G9 documents for Final Approval). Nevertheless, in the present study the vitality of the water was also determined as part of the environmental acceptability procedure. This was done by incubating treated water with freshly collected water from the Wadden Sea, containing the whole range of organisms

present in nature and not by means of a set of standard test organism, as typically is done in toxicology studies.

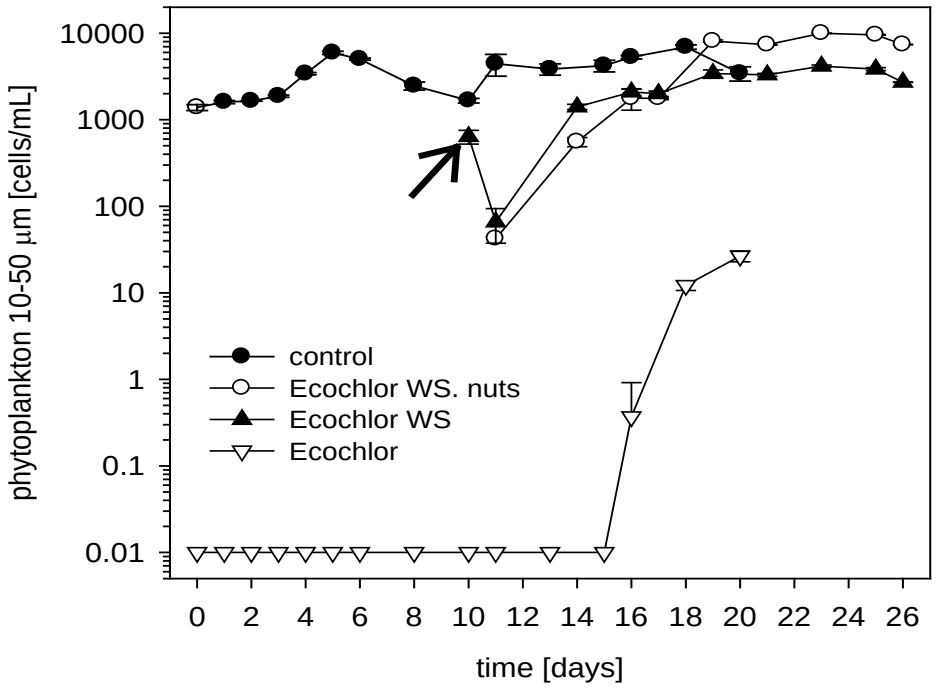


Figure 10: time course of phytoplankton numbers (10 – 50 µm size range) during a 26 day incubation period, control and treated water (Ecochlor). At day 11 a 10% (v/v) of Wadden Sea water was added to the treated water and incubated in separate bottle with (Ecochlor WS nuts) and without additional nutrients (Ecochlor WS). Arrow indicates the number of phytoplankton cells in the original sample prior to addition to the treated water.
Test run X; values are mean of three replicates

Figure 10 shows that immediately after the addition of freshly collected Wadden Sea water numbers of phytoplankton increased rapidly, irrespective of the nutrient conditions of the water. This was observed for all other phytoplankton fractions as well (data not shown). Within 7 days, phytoplankton numbers increased by two orders of magnitude reaching the same values as measured in the control bottle.

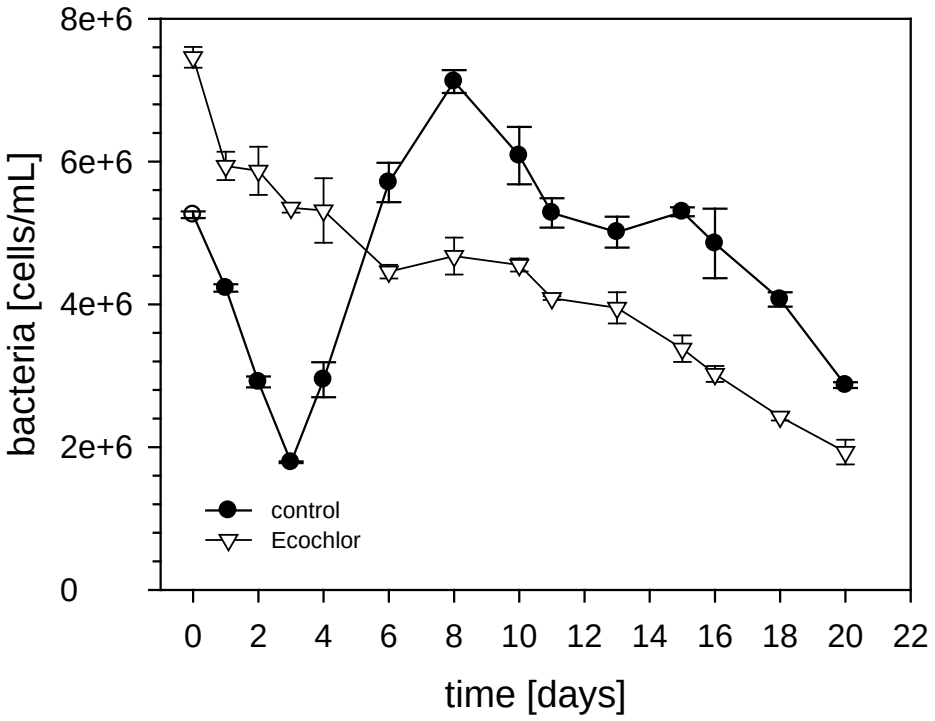


Figure 11: time course of bacteria numbers during a 20 day incubation period, control and treated water (Ecochlor). Test run X; values are mean of three replicates

With respect to bacteria numbers control and treated water showed a distinct different pattern (fig. 11). In the control bottle bacteria first declined by a factor two but this was followed by a period of regrowth in the subsequent 5 days. After day 8 numbers declined again to a final number which was less than at intake. In contrast bacteria numbers in the treated water declined throughout the whole period at an almost constant rate. At day 20 final numbers for both the control and treated water were nearly identical.

This gradual decline in the treated water implies that the active substance is actively reducing the number of bacteria like for the other organisms present. However, in contrast to phytoplankton which is immediately disintegrated after treatment bacteria remain present as intact cells and the degradation process takes far more time. Remarkable enough the regrowth of phytoplankton measured in test run 10 (Fig 9), starting at day 15, was not immediately followed by a growth response in bacteria.

10 Discussion and evaluation of results

The presented data show that the experimental design, type of test protocols used and additional experiments provide a solid data base of information on the performance of the ECOCHLOR® -BWT-System under semi-*in situ* conditions. The initial idea of a treatment system based on the use of the active substance chlorine dioxide alone turned out to be ineffective in waters with an extremely high density of hard-shell organisms like barnacles. Despite the fact that a high percentage of these organisms were killed, sufficiently high numbers survived the 5 day incubation period as viable organisms. To some extent this was a surprise since the numerous onboard tests conducted by the University of Rhode Island revealed that the system performed perfectly well without a coarse primary filter.
With only some minor deviations the present 11 test runs were conducted according to the IMO's G8 guidelines. The main conclusion is, as far as the biology is concerned, that the residual number of organisms at discharge are well in compliance with the Standard-D2. Often the numbers on discharge were at least one order of magnitude lower than the Standard-D2. The results of the various ecotoxicity studies and chemical analysis of treated water discharge are presented in the dossier for IMO G9 Final Approval.
The treated water remained stable for most of the parameters over the whole holding period of 5 days. Concentrations of dissolved oxygen (DO) and dissolved organic carbon (DOC) hardly varied. Only the total suspended solids and the particulate organic carbon declined slightly mainly as a result of sedimentation. In this respect it should be noted that the application of the self-cleaning filter effectively removed larger particles and the load of sediment in the treated tanks was minimal compared to the control tank.
The stable DO and DOC were indicative of a reduced biological activity. Indeed the treated water was not only void of organisms for the recommended period of 5 days but the long-term incubations also indicated that there was no regrowth of phyto- or zooplankton. This implies that the active substance is also actively preventing germination of resting stages, cysts or other type of dormant cells/organisms even under optimal growth conditions as applied in the climate rooms. Nevertheless, the treated water is far from 'dead' water. When a mixed plankton community from the Wadden Sea was added to the treated water the plankton community showed a rapid and unaffected growth response. It should be noted that the toxicological and chemical aspects of the treated water are discussed in detail in the environmental assessment studies as part of the IMO G9 application for Final Approval. Nevertheless, the present growth experiments, as part of the environmental acceptability aspects, clearly demonstrate that upon discharge there is no detrimental effect of the treated water to the organisms in the basin receiving this water in high quantities.

During the testing period we encountered some issues which need attention for future testing and legislation.

1. The number of organisms in the > 50 μm fraction, and to some extent also in the 10 – 50 μm size range, cannot (easily) be met in the salinity range > 32 PSU. Further, the addition of cultured organisms is very difficult and poses a variety of problems. With increasing salinity the seawater will have the characteristics of typical open ocean water. This implies low to extremely low numbers of the organisms in the above indicated size ranges. For that reason we have extended our focus to the organisms < 10 μm since there is no biologically relevant reasoning why tests should exclude this size fraction.
2. The long-term incubations (ca. 20 days) and the environmental acceptability studies provide good insight in the response of the whole community to the growth potential/limitation of the treated water when released into environment. These experiments should be conducted next to the standard set of toxicology and residual chemistry tests of the treated ballast water.

3. Testing for the presence of human pathogens strongly depends on the natural abundance of these microbes in the natural environment. Since these pathogens cannot be supplemented for health and safety reasons, accurate testing is therefore not possible. Moreover, viability tests of the total bacteria community showed that not all bacteria were effectively killed. Therefore, at least in theory, the human pathogens would remain a potential risk. This is a factor of concern for the land-based tests as well as for the ship-board trials when the ship remains in fairly clean ports for intake and discharge. In the case of ECOCHLOR® -system its active compound (chlorine dioxide) has been evaluated for total and faecal coliforms (*E. coli:*; (Oviatt *et al.*, 2002; URI unpublished on-going study) as well as with *V. cholerea* (Oviatt *et al.*, 2002; URI unpublished on-going study) and proven to be an effective method of disinfection.

In conclusion, the present configuration of the ECOCHLOR® -system offers a reliable and environmentally safe cleaning of the ballast water resulting in organism numbers well below the Standard of the IMO Regulation-D2.

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Appendix 1

Phytoplankton Marsdiep April-July 2008 ***data by Jolanda van Iperen***	***+: present but rare, ++: present; +++: dominant;*** ***++++: very abundant; +++++: massively present***	
group	***species name***	***relative dominance***
autotrophic flagellate	*Phaeocystis globosa* colony cell	+++++
	Phaeocystis globosa colonies < 50 µm	++++
	Phaeocystis globosa colonies > 50 µm	+++++
autotrophic flagellate	*Phaeocystis globosa flagellate cell*	+++++
heterotrophic flagellate	*parasite cyst of Ochromonas* group	++++
diatom pennate	*Pseudonitzschia delicatissima* group	++++
heterotrophic flagellate	*heterotrophic flagellate* indet. <10 µm	++++
autotrophic flagellate	*Prymnesiales* indet. <10 µm	++++
diatom centricate	*Thalassiosira* spp. 10 µm	++++
diatom centricate	*Chaetoceros socialis*	++++
autotrophic flagellate	*Hemiselmis* group	+++
diatom centricate	*Skeletonema "costatum"* group	+++
autotrophic flagellate	*Plagioselmis* group	+++
heterotrophic flagellate	*Paulinella* spp.	+++
heterotrophic flagellate	*heterotrophic flagellate* indet. 10-30 µm	++
autotrophic flagellate	*Pyramimonas* spp. <10 µm	++
heterotrophic flagellate	*Choanoflagellatea* indet.	++
hetero/auto flagellate	*Cryptophyceae* "light" group	++
autotrophic flagellate	*Teleaulax acuta* group	++
heterotrophic dinoflagellate	*Oxyrrhis marina*	++
diatom centricate	*Leptocylindrus minimus*	++
diatom centricate	*Minutocellus* group	++
diatom centricate	*Thalassiosira* spp. 10-30 µm	++
heterotrophic dinoflagellate	*Katodinium glaucum*	++
diatom centricate	*Guinardia delicatula*	++
freshwater green alga	*Pediastrum* spp.	++
heterotrophic flagellate	*Ciliophrys* group	++
autotrophic flagellate	*Prasinophyceae* indet. <10 µm	++
hetero/auto dinoflagellate	*Gymnodiniaceae* indet. 10-30 µm	++
diatom centricate	Various species	++
autotrophic flagellate	*autotrophic flagellate* indet. <10 µm	++
diatom centricate	*Chaetoceros* spp. <10 µm solitairy cells	+
autotrophic flagellate	*Chlorophyceae, Telonema* spp.	+
heterotrophic flagellate	*Chrysophyceae* indet.	+
freshwater green alga	*Crucigenia* spp., *Chlorophyta*	+
diatom centricate	*Skeletonema "costatum"* lenses	+
freshwater green alga	*Oocystis* spp.	+
diatom pennate	*Asterionellopsis glacialis*	+
autotrophic flagellate	*Rhodomonas* group	+
heterotrophic flagellate	*Bodo* group	+

Species list zooplankton and some larger (atypical) plankton

Appendix 1 continued

Phylum	Class	Subclass, Order, etc.	species no.	Identified genera	most likely present
Sarcomastigophora		Dinoflagellida	*2*	*Noctiluca, Protoperidinium*	
Bacillariophyceae			*3+*	*Bidulphia, Coscinodiscus*	
Cnidaria	Hydrozoa		*2+*	*Obelia*	
	Scyphozoa		*2*	*Aurelia, Cyanea*	
Ctenophora			*2+*		*Pleurobrachia, Beroe, Mnemiopsis*
Nemathelminthes	Rotatoria		*1+*		*Asplanchna*
	Nematoda		*1+*		
Annelida	Polychaeta		*2+*		
Arthropoda	Crustacea	Order Calanoida	*4+*	*Temora, Acartia, Centropages, Calanus and/or Pseudocalanus*	*Oithona*
		Order Harpacticoida	*2+*		*Tigriopus*
		Subclass Cirripedia	*1+*		*Semibalanus*
		Suborder Cladocera	*2*	*Podon, Evadne*	
		Subclass Malacostraca	*2+*	*Carcinus (zoea larvae)*	
Mollusca	Gastropoda		*1+*		*Littorina*
	Lamellibranchia		*2+*	*Cerastoderma*	*Mya*
Echinodermata	Ophiuroidea and/or		*2+*		*Ophiothrix, Echinocardium*
	Echinoidea				
Urochordata	Larvacea		*1*	*Oikopleura*	
Minimum number of species encountered (10 phyla):			*32*		